INOCULATION OF WHITE MICE WITH PFEIFFER'S BACILLUS

INFLUENZA STUDIES. IX *

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Some investigators have reported that Pfeiffer's bacillus is nonpathogenic for white mice, others that the organism can be recovered from the heart blood of a mouse only when injected with other bacteria, while yet others assert that even in pure culture it is distinctly pathogenic for these animals.

Spooner, Scott, and Heath,[1] using cultures isolated at Camp Devens early in the influenza outbreak of 1918, stated that Pfeiffer's bacillus is nonpathogenic for white mice, basing their conclusions on more than 100 intraperitoneal injections. A description of the technic and a statement of the amounts injected were not given.

Jacobson[2] was not able to kill white mice by intraperitoneal injections in amounts of one slant of Pfeiffer's bacillus in pure culture, and he concluded that the organism was nonpathogenic for white mice. But when he grew it "symbiotically," or mixed it with streptococci, the virulence was sufficiently raised for smaller doses to cause the death of this animal. Wolf[3] likewise declared that this organism did not invade the blood stream of the mouse even when large amounts of pure culture were injected intraperitoneally, and since he was never able to demonstrate the bacillus in the heart blood, he was of the opinion that in these cases death was due to intoxication. But when he injected a broth culture of streptococcus intraperitoneally or subcutaneously together with one slant of Pfeiffer's bacillus, a fatal septicemia followed, and both organisms were recovered from the heart blood. Roos[4] obtained similar results in experiments on simultaneous intraperitoneal injections into white mice of Pfeiffer's bacillus with streptococcus or with sputum. The exact dosage used is not recorded. After such injections, Pfeiffer's bacillus was often demonstrable in the heart blood, and Roos thought that "the symbiosis of these organisms increased the virulence of B. influenzae about ten-fold."

Albert and Kelman[5] agree with Roos on the question of increase of virulence of Pfeiffer's bacillus in mixed culture with streptococcus or pneumo-

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[1] Jour. A. M. A., 1919, 72, p. 155.

[2] Arch. de Med. Exper., 1901, 13, p. 425.

[3] Centralbl. f. Bakteriol., I, O., 1920, 84, p. 241.

[4] Jour. Immunol., 1919, 4, p. 189.

[5] Jour. Infect. Dis., 1919, 25, p. 433.

coccus, but consider the organism even in pure culture to be distinctly pathogenic for white mice. They base their conclusion on series of 24 mice, among which there was a mortality of 83% following intraperitoneal injections, the amount injected not being stated. Similar results were reported by Ferry and Houghton,[6] who found that the strains used in their investigation of the toxin of Pfeiffer's bacillus all proved pathogenic for white mice, and that these animals were "invariably susceptible." Wollstein,[7] found that mice were highly susceptible to small injections of either spinal fluid of influenzal meningitis or pure cultures therefrom. The 8 cases reported by her at that time were all fatal, and hence the virulence of the strains may be considered high.

These differences in the findings as to pathogenicity by various workers may be due to differences in the dosages used, as Albert and Kelman [5] conclude, but they might also conceivably be due to differences in the source of the cultures and, perhaps as a result, the virulence of the strains.

The experiments reported here were undertaken to throw some light not only on the pathogenicity of Pfeiffer's bacillus for white mice, but also to relate the pathogenicity to the source of the strain. Other questions investigated were the invasiveness of the bacillus in pure and mixed culture, the possibility of increase in virulence by passage through white mice, and the presence of immunity in those mice that have recovered from a sublethal dose.

The strains used in these experiments were obtained from various sources. They were repeatedly examined and identified in this laboratory as Pfeiffer's bacillus. They had been cultivated on chocolate agar for varying lengths of time, although they had been isolated within a period of five months. In a short preliminary investigation it was found that the length of cultivation on artificial mediums within the limits of several weeks had no marked effect on the comparative pathogenicity of the bacillus for white mice, comparing strains from the same source. This allowed for reliable grouping of results from strains of one source and also for comparison of results from strains of different sources. All injections were made intraperitoneally. In the experiments on pathogenicity a 22-24 hour culture on chocolate agar slant suspended in 1 c c saline solution was injected into each mouse. A standard slant was adopted and adhered to closely. It was a slant about 8 cm. long in an ordinary 5/8 by 6 inch test-tube. An even growth was obtained by gently smearing the entire surface of the slant when inoculating.

[6] Jour. Immunol., 1919, 4, p. 233.
[7] Am. Jour. Dis. Child., 1911, 1, p. 42.

Early in the investigation on pathogenicity it was found that white mice had considerable individual susceptibility or resistance to this organism, and hence the plan was adopted of injecting the same strain into a series of 5 mice. This could not be done in every case because of the loss in the laboratory stock of some of the cultures before the adoption of this scheme and before the series of 5 mice for each strain was complete. This had little effect, however, on the final results as table 1 shows. One slant each of the 44 strains used was injected intraperitoneally into 146 mice, of which 120 died with the resulting mortality of 82%, which is in near agreement with the mortality of 83% which Albert and Kelman [5] found in a series of 24 mice similarly injected. The average period of survival among the 120 mice that died was about 19 hours. Considering only those strains that were injected in a series of 5 mice (4 mice in one case), we find nearly the same results, namely a mortality of 80% among 119 mice injected with 24 strains, and an average period of survival of about 18 hours after injection. It is worthy of note that one strain of Pfeiffer's bacillus isolated from a case of influenzal meningitis killed 3 out of 5 mice injected in the routine manner. The average period of survival was about 25 hours. This strain had been cultivated on artificial mediums for the same length of time as the other strains, and, although the results are comparable, they are not included in the figures presented since this was the only strain from influenzal meningitis.

Heart blood cultures of the mice that died usually showed the presence of Pfeiffer's bacillus either in pure culture or mixed with a postmortem invading organism, which was commonly a bacillus of the colon group. Pfeiffer's bacillus was recovered in pure culture from the heart blood in 68%, and either in pure culture or with another organism in 86%, of the fatal cases. The presence of Pfeiffer's bacillus was determined by cultivating the heart blood on chocolate agar and identifying the bacillus by its colony, morphology, and staining reaction. The peritoneal fluid was cultivated regularly also and Pfeiffer's bacillus was often found in the peritoneal fluid in pure culture, but another organism usually belonging to the colon group occurred with it about twice as often as in the heart blood. Gram-positive cocci were rarely found in the cultivation of either the heart blood or peritoneal fluid, being found 4 times in the cultures from 146 mice. Hence these cocci could not be considered an important factor in causing the death of the mice. These cultural findings are contrary to those of Wolf [3] who reported a repeatedly sterile heart blood even in fatal cases following

injections of pure cultures. The high mortality following intraperitoneal injections of pure cultures of Pfeiffer's bacillus and the recovery of the same organism from the heart blood favor the view that the Pfeiffer's bacillus invades the blood stream and is pathogenic for white mice, irrespective of the source.

The original cultures came from several sources: throats of normal persons, throats of persons with common colds, and throats of military

TABLE 1

RESULTS OF INJECTIONS OF STRAINS OF PFEIFFER'S BACILLUS FROM VARIOUS SOURCES

I. Strains Tested in 1 or More Mice

Source of Original Cultures	Number of Cases from Which Strains Came	Number of Strains Tested in 1 or More Mice	Total Number of Mice Injected with All Strains	Number of Mice That Died	Number That Recovered	Percentage That Died	Average Hours of Survival After Injection
1. Normal respiratory tract.....	8	10	31	25	6	80	19.8
2. Persons with common colds.....	6	7	35	31	4	88	19.2
3. Influenza cases—civilian.....	7	8	36	22	14	61	22.4
4. Influenza cases—military.....	8	19	44	42	2	95	16.0
Totals.....	29	44	146	120	26	82	19.3

II. Strains Tested in Series of 5 Mice Each

Source of Original Cultures	Number of Cases from Which Strains Came	Number of Strains Tested in Series of 5 Mice	Total Number of Mice Injected in Series	Number of Mice That Died	Number That Recovered	Percentage That Died	Average Hours of Survival After Injection
1. Normal respiratory tract.....	4	4	20	16	4	80	18.1
2. Persons with common colds.....	6	7	35	31	4	88.6	19.2
3. Influenza cases—civilian.....	7	7	35	21	14	58	23.0
4. Influenza cases—military.....	5	6	29	28	1	96.5	13.8
Totals.....	22	24	119	96	23	80.6	18.5

and civilian patients with influenza. The observations gave an opportunity to determine the relation of the source of the strain to its pathogenicity for white mice. Correlation of the results of these experiments, as indicated in table 1, shows that the strains from the influenza epidemics at the Great Lakes Training Station and at Camp Grant early in 1920 were the most highly pathogenic with a mortality of 96.5% and an average period of survival of only 13 hours among 29 mice injected. The strains from persons with common colds, from

normal throats, and from throats of influenza patients among civilians followed in that order in decreasing mortality rate and a corresponding lengthening of the period of survival after injection. While the evidence here is interesting, and indicative of the high virulence of strains of Pfeiffer's bacillus coming from epidemics in military camps, it cannot be taken as meaning that there is a definite etiologic relation of this organism to such an epidemic; nor can it be taken as conclusive that the virulence of Pfeiffer's bacillus is increased in epidemics of influenza, since the strains from typical civilian cases of influenza furnished the lowest mortality rate among white mice with the longest average period of survival. On the other hand, the conditions in the military camps, especially during a respiratory epidemic, were conducive to rapid transfer of respiratory organisms, which might tend to increase their virulence irrespective of the existence of an influenza epidemic. Likewise this might explain the lower virulence of the strains from civilian cases of influenza. The less crowded conditions in the civilian populace would give less opportunity for rapid transfer of respiratory organisms. The small difference between the mortality of mice injected with strains from normal throats and from throats of persons with common colds is not enough to give any significance to the presence of more virulent strains of Pfeiffer's bacillus in connection with common colds. The experimental evidence on these points is summarized in table 1.

Experiments were made to show the result of simultaneous injections of Pfeiffer's bacillus together with Streptococcus viridans and the pneumococcus. Strains of these cocci were taken at random from the laboratory stock cultures and had been grown on artificial mediums for some time (at least several months). Amounts of these gram-positive cocci were standardized for injection by growing them on whole blood-agar slants of the same size as for Pfeiffer's bacillus.

Streptococcus viridans was injected in amounts of 1 slant and one-half slant with the same doses of Pfeiffer's bacillus. The pneumococcus was injected in one-half and one-eighth slant amounts with equal doses of Pfeiffer's bacillus. The control and mixed culture tests of the pneumococcus and the bacillus were made in series of at least 5 mice of each amount, and the results presented are averages of such tests (table 2).

In no instance did the mouse survive when injected with both Pfeiffer's bacillus and a gram-positive coccus. Both organisms were invariably isolated from the heart blood in approximately equal num-

bers, although one-eighth slant of the same strain of Pfeiffer's bacillus used in pure culture was previously found always sublethal. The control mice injected with both amounts of Streptococcus viridans alone recovered. Pure cultures of pneumococcus were repeatedly fatal, but the simultaneous inoculations with Pfeiffer's bacillus were more rapidly fatal.

Streptococcus viridans was recovered from the heart blood of mice inoculated with mixed cultures of the coccus and Pfeiffer's bacillus, although the same dosages of the coccus in pure culture were innocuous. Likewise, sublethal doses of Pfeiffer's bacillus (one-eighth slant) with

TABLE 2

SIMULTANEOUS INJECTIONS OF PFEIFFER'S BACILLUS WITH STREPTOCOCCUS VIRIDANS AND PNEUMOCOCCUS TYPE 3

	Amount Injected, in Slants	Period of Survival After Injections of Pure Cultures	Period of Survival After Injections of Pfeiffer's Bacillus with Gram-positive Coccus in Equal Amounts	Findings in Culture of Heart Blood Following Injections of Mixed Cultures
Pfeiffer's bacillus.....	1	16 hours		
	½	22 hours		
	⅛	All recovered		
Streptococcus viridans..	1	Recovered	14 hours	Pfeiffer's bacillus and Streptococcus viridans
	½	Recovered	30 hours	Pfeiffer's bacillus and Streptococcus viridans
Pneumococcus Type III.	½	13 hours	11 hours	Pfeiffer's bacillus and pneumococcus
	⅛	18 hours	11 hours	Pfeiffer's bacillus and pneumococcus

the same amount of pneumococcus resulted in the death of all the mice so injected and in the recovery of both organisms from cultures of heart blood. It seems from these experiments that the invasiveness of both Pfeiffer's bacillus and Streptococcus viridans was increased by simultaneous mixed inoculations. A definite conclusion cannot be drawn, however, as to whether the coccus or the bacillus had the more active part in causing the death of the mouse since they were found on culture in approximately equal numbers.

The question whether an increase in virulence of Pfeiffer's bacillus was produced by passage through white mice was studied by a series of injections in decreasing doses of the heart blood culture of a fatal case. In the first group of experiments, 11 mice were fatally injected each with 1 slant of stock cultures. One-half slant of pure cultures

from the heart blood of these 11 mice was injected into 11 other mice. As a result, only 4 mice succumbed with an average period of survival of 14 hours, while the 11 original mice injected with full slants had averaged about 13 hours of survival. The virulence was not sufficiently raised in one passage for half the original dose to be regularly fatal. In another group of experiments, repeated injections were made of pure cultures from the heart blood of the mouse dying with the minimal lethal dose and at the same time furnishing a pure culture of the bacillus in the heart blood. Decreasing doses of the pure subculture were given to several mice, fractions of a slant being injected as parts of a salt suspension of that slant. Variations in results presented themselves which were probably due to differences in resistance and susceptibility of the mice. The lethal amount of bacterial suspension was not reduced to any marked extent by such repeated transfers. Each series of tests was blocked after 3 or 4 transfers, however, either by inability to recover Pfeiffer's bacillus from the heart blood, or failure to recover it in pure culture.

These results are comparable to those of Roos [4] who found that the virulence of this organism is not increased by passage through rabbits. They also agree with the findings of Wollstein [7] who reported that "it was not found possible to increase the virulence appreciably by passing the organisms through series of mice, although the average dose was found to be reduced in the ninth passage to about one half the original fatal dose." Ferry and Houghton,[6] on the other hand, found the virulence "increased four-fold, and over" by repeated injections. The kind of animal used, however, is not specifically named. Jacobson [2] found that by repeatedly injecting mixed cultures of Pfeiffer's bacillus and streptococcus, he recovered from the heart blood, after 5 or 6 transfers, the Pfeiffer's bacillus which had increased in virulence sufficiently to kill a mouse even in pure culture, whereas originally the same strain was nonlethal. The brief experiments summarized in table 3 suggest that there was no marked increase in the virulence of Pfeiffer's bacillus by passage through white mice as many as 3 or 4 times. The arrow in the table indicates the mouse from whose heart blood was cultivated the bacillus for injection into the next group in the stated amounts. The hours refer to the period of survival after injection.

Observations were made on the degree of immunity possessed by those mice that had recovered from sublethal doses of Pfeiffer's bacillus. Eleven mice recovered from injections of sublethal doses of

strains picked at random from the stock cultures on hand. The doses varied from 1/16 to 1/4 slant in amount. To these mice was added a control mouse that recovered from a dose lethal to 5 other mice. These 12 mice were then inoculated with doses found to be lethal to control mice. One-sixteenth to 1/4 slant of the strains used were sublethal and immunizing in effect, and 1/2 slant generally lethal to the nonimmunized mice. Seven of the 12 mice were injected with lethal and then twice

TABLE 3

EXPERIMENTS ON THE INCREASE IN VIRULENCE OF PFEIFFER'S BACILLUS BY PASSAGE THROUGH WHITE MICE

Strain	Amount Injected				
	1 Slant	½ Slant	¼ Slant	⅛ Slant	1/16 Slant
GL 6 NPBc	9 hours ↓				
	10 hours	10 hours	5 hours ↓		
		24 hours ↓	Recovered	Recovered	Recovered
	17 hours	17 hours	29 hours ↓	Recovered	Recovered
	17 hours	36 hours	Recovered	1 hour*	Recovered
GL 1 TBc	4 hours ↓				
	25 hours ↓	12 hours	Recovered	Recovered	Recovered
	26 hours	11 hours	Recovered	Recovered	Recovered
GL 10 TBc	26 hours	19 hours ↓			
	8 hours ↓	Recovered	Recovered	Recovered	Recovered
	21 hours ↓	Recovered	Recovered	Recovered	Recovered
	42 hours	26 hours	Recovered	Recovered	Recovered

* Died in convulsions at end of 1 hour.

lethal doses of the same strain. Six of the 7 mice recovered from the lethal dose, and 5 of the remaining 6 recovered from the twice lethal dose. These 5 mice and the other 5 of the original 12 immunized mice were then injected successively with 2 other strains of Pfeiffer's bacillus taken at random from the stock cultures. The dose of the first strain was lethal and of the second strain twice lethal. All the mice but 1 recovered from these two injections. Several control injections of these 2 strains were fatal in every case. The injections

throughout the experiments on immunity were made at intervals of a few days and extended over a period of 40 days. The immunized mice showed no signs of sickness when injected with lethal doses and were plainly protected by previous sublethal doses. The mice were protected not only against the strain used in immunization, but they possessed immunity also against lethal doses of other strains. During the following 8 weeks, 2 of the immunized mice died of unknown cause. At the end of that time the 6 remaining mice were injected with a twice lethal dose of one of the immunizing strains. Only 1 mouse succumbed. This indicated that the specific and cross immunity lasted at least for the period of 8 weeks. The results of these experiments are given in table 4.

TABLE 4

EXPERIMENTS ON IMMUNITY ACQUIRED BY WHITE MICE AGAINST PFEIFFER'S BACILLUS

Strain.....	GL 10 TBc						GL 6 TOa	N 18 NPOb		N 9 NPOb	C 225 NPB	GL 10 TBc
Nature of dose.	Immunizing			Lethal		Twice Lethal	Lethal	Immunizing	Lethal	Lethal	Twice Lethal	Twice Lethal
Amount of dose in slants.....	¼	⅛	1/16	½	½	1	½	⅛	½	½	1	1
Mouse 284.....	L	..	..	L	L	L	L	..	..	L*		
Mouse 285.....	L	..	..	L	L	L	L	..	..	L	L	D
Mouse 272.....	L	..	..	L	L	L	L	..	..	D		
Mouse 277.....	L	..	..	D								
Mouse 273.....	..	L	..	L	L	D						
Mouse 278.....	..	L	..	L	..	L	L	..	..	L	L	L
Mouse 274.....	..	..	L	L	L	L	L	..	..	L	L†	
Mouse 296.....	..	..	..	..	..	..	..	L	..	L	L	L
Mouse 297.....	..	..	..	..	..	..	..	L	..	L	L	L
Mouse 298.....	..	..	..	..	..	..	..	L	..	L	L	L
Mouse 299.....	..	..	..	..	..	..	..	L	..	L	L†	
Mouse 291..... (a control that lived)	..	..	..	..	..	..	..	L	L	L	L	L
Control mice—												
Died.....	..	..	..	10	..	5	4	..	..	5	5	10
Recovered....	..	..	..	1	..	0	1	..	..	0	0	1

L = lived; D = died.
* 284 died of unknown cause 4 days after complete recovery from previous injection.
† 274 and 299 died several weeks after complete recovery from previous injection.
Note: Eight weeks elapsed between the injections of C 225 NPB and GL 10 TBc.

SUMMARY

Pfeiffer's bacillus when injected intraperitoneally in pure culture was found to be pathogenic for white mice irrespective of the source, and was readily recovered from the heart blood by cultivation on chocolate-agar medium. Strains isolated during influenza epidemics at military camps were more pathogenic for white mice than strains from other sources.

The invasiveness of both Pfeiffer's bacillus and Streptococcus viridans seemed to have been increased by injections in mixed cultures: the bacillus by injection with pneumococcus, and the coccus by injection with Pfeiffer's bacillus.

Pfeiffer's bacillus was not found to be appreciably increased in virulence by passage three or four times through white mice.

Sublethal doses (one-fourth to one-sixteenth slant of the strains used) of Pfeiffer's bacillus conferred immunity to white mice against lethal (one-half slant) and twice lethal (one slant) doses of heterologous as well as homologous strains. This immunity lasted at least eight weeks.